# Endophytic fungi associated with leaves of Betulaceae in Japan.

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**Abstract:** Diversity and species composition of endophytic fungi on leaves of 11 tree species in Betulaceae were studied, with reference to climatic, tree species, and seasonal variations. A total of 186 fungal isolates were obtained from 190 leaves collected in a subalpine, a cool temperate, and a subtropical forest in Japan and were divided into 46 operational taxonomic units (OTUs) according to the base sequences of D1/D2 region of LSU rDNA. The two most frequent OTUs were *Muscodor* sp. and *Nemania* sp. in Xylariaceae, followed by *Gnomonia* sp., *Glomerella acutata*, *Apiosporopsis* sp., *Asteroma* sp., and *Cladosporium cladosporioides*. The similarities of OTU composition in endophytic

fungal assemblages on leaves of Betulaceae were generally low among the forests of different climatic regions. Fungal OTU compositions were relatively similar between two *Betula* species in subalpine forest, whereas seven tree species in cool temperate forest were divided into three groups according to the similarity of endophytic fungal assemblages on the leaves, with four *Carpinus* species assigned into two of the three groups. The similarity of endophytic fungal assemblages between August and October was relatively high in subalpine forest, whereas the seasonal changes were generally greater (i.e. the similarities among sampling dates were lower) in cool temperate forest.

*Key words*: Betulaceae, climatic region, endophyte, seasonal change, tree leaves.

## Introduction

Endophytic fungi include those that live within plant tissues at some time in their life without causing apparent harm to their host (Saikkonen 2007; Sieber 2007). Endophytic fungi on leaves of Betulaceae have been studied mainly in Europe and north America, with emphasis on species richness (Sieber et al. 1991; Barengo et al. 2000; Green 2004), genetic diversity (Lappalainen and Yli-Mattila 1999), relationships with genetic variations of host

trees (Elamo et al. 1999; Ahlholm et al. 2002b; Saikkonen et al. 2003), interactions with herbivores (Lappalainen and Helander 1997; Lappalainen et al. 1999; Ahlholm et al. 2002a; Valkama et al. 2005), and effects of simulated acid rain (Helander and Rantio-Lehtimäki 1990; Helander et al. 1993a, 1993b). In contrast, basic information is still lacking on the diversity and ecology of endophytic fungi of Betulaceous trees in Japan and Asian region, despite their occurrence as major components of various forest types including alpine, boreal, subalpine, temperate, and subtropical forests. Previous studies in Japan have examined leaves of a total of 182 tree species in 54 families and detected 24 fungal genera as endophytes (summarized in Osono 2009). Trees of Betulaceae have not been explored so far regarding foliar fungal endophytes and are expected to host further array of fungal diversity and functioning.

The purpose of the present study was to investigate the diversity and species composition of endophytic fungi on leaves of 11 tree species in Betulaceae, with reference to climatic, tree species, and seasonal variations. Endophytic fungi were isolated from healthy-looking leaves collected in a subalpine, a cool temperate, and a subtropical forest in Japan by means of a surface disinfection method and used for molecular phylogenetic analysis to determine putative taxa according to base sequences of D1/D2 region of LSU

rDNA and to classify them into operational taxonomic units.

## Materials and methods

### Study site

Leaves of Betulaceous trees were collected at three study sites located in different climatic regions in Japan: a subalpine, a cool temperate, and a subtropical forest. The subalpine forest is located on Mt. Ontake, Gifu, central Japan (35°56'N, 137°28'E, altitude 2050 m). The cool temperate forest is located in Ashiu Experimental Forest of Kyoto University, Kyoto, central Japan (35°18'N, 135°43'E, altitude 660 m). The subtropical forest is located in Yona Experimental Forest of University of the Ryukyus in the northern part of Okinawa Island, Okinawa, south-western Japan (26°9'N, 128°5'E, altitude 250 m). Mean annual temperature of the study sites is approximately 2°C, 10°C, and 22°C in subalpine, cool temperate, and subtropical forest, respectively, and annual precipitation is 2500 mm, 2353 mm, and 2456 mm, respectively. Details of the three study sites are described in previous papers of litter-decomposing microfungi by the first author (Osono 2002; Osono and Takeda 2007; Osono et al. 2008). Snow covers the forest floor from November to May

in subalpine and from December to April in cool temperate forest, whereas no snow cover has been observed in subtropical forest.

**Sample collection**

Leaves of a total of 11 deciduous tree species in four genera of Betulaceae were collected in the present study, three species in the subalpine forest, seven in the cool temperate forest, and one in the subtropical forest (Table 1). The 11 tree species are major components of natural forest stands in the respective study sites. The collection was performed during the growing season from May to November in 2008, once or three times per tree species, according to Table 1. On each sampling occasion, a total of 10 healthy-looking leaves were harvested for each tree species from two arbitrarily chosen trees growing naturally in forests, two branches per individual tree, at an approximate height of 3-4 m. This made the harvest of a total of 190 leaves during the study period. The leaves were placed in paper bags and taken to the laboratory.

One leaf disk was punched out from the central part of each sample leaf, avoiding the primary vein, with a sterile cork borer (5.5 mm in diameter). A total of 10 leaf disks were used to isolate endophytic fungi for each tree species and on each sampling occasion.

Fungi were isolated within 24 h of collection in the cool temperate forests and within 48 h in the subalpine and subtropical forest.

**Fungal isolation**

A surface disinfection method (Kinkel and Andrews 1988) was used to isolate endophytic fungi. The leaf disks were submerged in 70% ethanol (v/v) for 1 min to wet the surface, then surface disinfected for 30 s in a solution of 15% hydrogen peroxide, and then submerged again for 1 min in 70% ethanol. The disks were rinsed with sterile, distilled water, transferred to sterile filter paper in Petri dishes (9 cm in diameter), and dried for 24 h to suppress vigorous bacterial growth after plating (Widden and Parkinson 1973). The disks were placed on 9 cm Petri dishes containing lignocellulose agar (LCA) modified by Miura and Kudo (1970), two disks per plate. LCA contains glucose 0.1%, $KH_2PO_4$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02%, and agar 1.3% (w/v). Note that LCA modified by Miura and Kudo (1970) does not contain lignin or other recalcitrant compounds. The modified LCA was used because its low glucose content suppresses the overgrowth of fast-growing fungal species (Osono and Takeda 1999). Plates were incubated at 20°C in the dark and observed at 1, 4, and 8 weeks after surface

disinfection. Any hyphae or spores on the plates were subcultured on fresh LCA to establish pure cultures.

**Determination of OTUs**

Obtained pure cultures were identified by morphology and molecular analysis. Where fungal structures such as spores and sporocarps were produced on the medium, their morphological characteristics were observed by using Nikon Optiphot microscope (Nikon Inc. Tokyo). However, on many cultures, the fungal structures have not been seen. Thus, identification of the cultures also was attempted by molecular analysis. Small amount of mycelial tips from each culture were picked, crushed in the 24 µl distilled water in the tube, and microwaved at 12 seconds. They were used as templates for PCR. Fifty µl of reaction mixture containing 25 µl Qiagen GoTaq premix and 10 pmol of each primer and distilled water were added to the templates. The oligonucleotide primer-pair, NL1 and NL4 (White et al. 1990) were used for PCR of ribosomal DNA large subunit D1/D2 region. The reactions were initiated with 4 min of denaturation at 95°C, followed by 40 cycles of two-step PCR, consisting of 20 s at 94°C and 60 s at 56°C with final extension for 10 min at 72°C on a GeneAmp 9700 thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, California).

Amplification products were purified by QIAquick PCR Purification Kit (QIAGEN), and used for sequencing with a Big Dye Terminator Cycle Sequencing FS Ready Reaction kit ver. 3.1 and ABI PRISM 3100 genetic analyzer (Perkin-Elmer Applied Biosystems). Both strands of a fragment were sequenced. Sequence data sets were manually truncated both ends and edited using the program BioEdit sequence editor version 5.09 (Hall 1999). Homology searches were performed using each obtained sequences data on a BLAST program at the National Center for Biotechnology Information (NCBI). Neighbor joining trees were also constructed using MEGA version 5 (Tamura et al. 2011) with related sequences from NCBI database. Isolates with more than 99% homology of sequence and within the same cluster were treated as OTUs (operational taxonomy units) with tentative codes for data analysis. In the case that obtained sequences contained polymorphic sites, they are treated as the same OTUs with close relatives.

**Data analysis**

Frequency of occurrence of a single fungal OTU was calculated as the percentage of the number of disks containing the fungal OTU out of the 10 disks tested for each tree species on each sampling occasion. Fungal OTUs were regarded arbitrarily as major ones

when equal to or more than five isolates were obtained during the investigation.

The degree of similarity of OTU composition among endophytic fungal assemblages at different sites, tree species, and seasons was examined using Pianka's α (Pianka 1973), which is expressed by the following formula:

$$\alpha_{1,2} = \sum_{i=1}^{S} p_{1i}\, p_{2i} \Big/ \sqrt{\sum_{i=1}^{S} p_{1i}^{\,2} \sum_{i=1}^{S} p_{2i}^{\,2}}$$

where $\alpha_{1,2}$ represents the similarity between assemblages 1 and 2, $P_{1i}$ and $P_{2i}$ are the proportions of the *i*th species in assemblages 1 and 2, respectively, and S is the total number of species. For $\alpha$=1, the two assemblages are identical in terms of their OTU composition.

Principal component analysis was used to ordinate endophytic fungal assemblages on leaves of Betulaceae, using data for 11 major fungal OTUs, with JMP ver. 6.0 software (SAS Institute 2005). The data of *B. ermanii* in June and *A. firma*, *B. grossa*, and *C. laxiflora* in May were not included in the analysis because of the low number of fungal isolates obtained from these samples.

## Results

### Operational taxonomic units of endophytic fungi

Zero to 17 isolates of endophytic fungi were obtained from leaves of each tree species of Betulaceae each sampling occasion, making a total of 186 isolates from 190 leaves (Table 1). The 186 isolates were divided into 46 OTUs according to their base sequences of D1/D2 region of LSU rDNA (Table 2), with 0 to 13 OTUs being recorded per tree species per sampling occasion (Table 1). Twenty-one (46%) of the 46 OTUs were singletons (Table 2). No endophytic fungi were isolated from *Betula ermanii* in June (Table 1). Most of the OTUs had sequences similar to those registered in GenBank database with the similarity score of 95-99% and were identified to genus or species (Table 2). The two most frequent OTUs were *Muscodor* sp. 11Bg52 and *Nemania* sp. 8Cs51 in Xylariaceae, followed by *Gnominia* sp. 11Af11, *Glomerella acutata*, *Apiosporopsis* sp. 11Af21, *Asteroma* sp. 8Ah91, and *Cladosporium cladosporioides* (Table 2).

**Frequency of occurrence of major OUT**

*Muscodor* sp. 11Bg52 occurred on seven tree species in subalpine and cool temperate forests and was frequent on *Alnus firma*, *Carpinus tschonoskii*, and *C. japonica* in cool temperate forest in August (Table 3). *Nemania* sp. 8Cs51 occurred on seven tree species in subalpine, cool temperate, and subtropical forests and tended to be more frequent

on warmer regions (Table 3). *Gnomonia* sp. 11Af11 occurred on *B. ermanii* and *B. platyphylla* var. *japonica* in subalpine forest (Table 3). *Glomerella acutata* occurred on five tree species in subalpine and cool temperate forests and was frequent on *B. grossa* and *C. cordata* in cool temperate forest in August (Table 3). *Apiosporopsis* sp. 11Af21 occurred on four tree species in cool temperate forest and was frequent on *A. firma* in November (Table 3). *Asteroma* sp. 8Ah91 occurred on three tree species in subalpine forest. *Cladosporium cladosporioides* occurred on six tree species in cool temperate (in August) and subtropical forests (in July). *Khuskia* sp. 11Bg11 and *Nigrospora* sp. 11Bg31 occurred on *B. grossa* and *C. laxiflora* in November. *Mycosphaerella* sp. 8Ah31 occurred on *A. hirsuta* var. *sibirica* in August. *Pestalotiopsis* sp. 8Ch61 occurred on *C. sieboldiana* in August.

**Similarity among climatic regions**

Similarity of endophytic fungal assemblages among climatic regions was examined for leaves of the same tree genera collected on relatively close dates (Table 4). The comparison of endophytic fungal assemblages on *Alnus* leaves in July to August gave relatively low Pianka's similarity indices of 0.03 to 0.30 for three forest types, with the greatest difference between subalpine and cool temperate forests (Table 4). This difference

was consistent with the results of comparison on *Betula* leaves collected in August or in October to November between subalpine and cool temperate forests, giving the similarity indices of 0.00 to 0.07 (Table 4). Thus, the similarities of OTU composition in endophytic fungal assemblages on leaves of Betulaceae were generally low among forest types in different climatic regions.

**Similarity among tree species**

In subalpine forest in August, the similarity between two *Betula* species was much higher than those between *A. hirsuta* var. *sibirica* and two *Betula* species (Table 5). In contrast, such similarity in endophytic fungal assemblages within a single tree genus was not necessarily the case in cool temperate forest in August, in which seven tree species were classified into three groups (Groups 1, 2, and 3) according to the similarity of OTU composition of endophytic fungal assemblages (Table 5). Group 1 included *A. firma*, *C. japonica*, and *C. tschonoskii* with Pianka's similarity indices of 0.68 to 0.82 (Table 5). *Muscodor* sp. 11Bg52 was the most frequent OTU on the leaves of three tree species in Group 1 (Table 3). Group 2 included *B. grossa*, *C. laxiflora*, and *C. cordata* with Pianka's similarity indices of 0.65 to 0.84 (Table 5). *Glomerella acutata* was the most frequent OTU

on the leaves of three tree species in Group 2 (Table 3). The similarities of endophytic fungal assemblages between leaves of Groups 1 and 2 were relatively low (0.00 to 0.35) (Table 5). Group 3 included *Corylus sieboldiana*. *Pestalotiopsis* sp. 8Ch61 was the most frequent OTU on *C. sieboldiana* leaves, followed by *G. acutata* (Table 3). Endophytic fungal assemblages of this tree species had the similarity indices of 0.00 to 0.05 to Group1 and 0.39 to 0.46 to Group 2 (Table 5). The similarity indices in cool temperate forest in November were consistent with the results in August, with the higher similarity between *B. grossa* and *C. laxiflora* in Group 2 than those between *A. firma* in Group 1 and two Group 2 tree species (Table 5).

**Similarity among seasons**

Similarity of endophytic fungal assemblages was relatively high (0.82) between August and October for *B. ermanii* leaves in subalpine forest (Table 6). In contrast, the similarity among seasons was generally low in cool temperate forest, with Pianka's similarity indices between 0.00 and 0.19 (Table 6).

**Principal component analysis**

The first two principal components (PC1 and PC2) accounted for 46.5% of total variance in the data for the 11 major endophytic fungal OTUs. PC1 (eigenvalue of 2.58) accounted for 23.5% of total variance and characterized endophytic fungal assemblages on leaves of Groups 2 and 3 in cool temperate forest in August (Fig. 1). PC2 (eigenvalue of 2.53) accounted for 23.0% of total variance and separated endophytic fungal assemblages in cool temperate and subtropical forests from those in subalpine forest (Fig. 1). Component loading of the major OTUs indicated that PC1 was positively associated with *Cladosporium cladosporioides*, *Glomerella acutata*, and *Pestalotiopsis* sp. 8Ch61 and negatively with *Asteroma* sp. 8Ah91, *Khuskia* sp. 11Bg11, *Nigrospora* sp. 11Bg31, *Mycosphaerella* sp. 8Ah31, and *Gnomonia* sp. 11Af11 (Fig. 1). PC2 was positively associated with *Nigrospora* sp. 11Bg31, *Khuskia* sp. 11Bg11, *Nemania* sp. 8Cs51, and *Muscodor* sp. 11Bg52 and negatively with *Asteroma* sp. 8Ah91, *Gnomonia* sp., 11Af11 and *Mycosphaerella* sp. 8Ah31 (Fig. 1).

## Discussion

Previous studies of diversity of endophytic fungi in Japan focused mainly on

leaves of trees in Ericaceae, Fagaceae, and pines (summarized in Osono 2009). Application of a molecular phylogenic analysis to fungal isolates from leaves of 11 trees in Betulaceae provides insights into putative taxonomy of fungal isolates including sterile mycelia, host relationship, and seasonal changes of fungal OTUs from different climatic regions.

The frequent occurrence of Xylariaceaeous endophytes (*Muscodor* sp. 11Bg52 and *Nemania* sp. 8Cs51, Table 2) on leaves of Betulaceae is consistent with previous studies of endophytic fungi in other tree leaves in Japan (Okane et al. 1997; Osono 2002, 2008; Osono and Mori 2004; Osono et al. 2008). Recent studies have demonstrated that endophytic species of *Muscodor* produce volatile organic compounds with antimicrobial and insecticidal activities (Daisy et al. 2002; Ezra et al. 2004; Lacey and Neven 2006). *Gnomonia* and *Glomerella* (Table 2), as anamorphic *Discula* and *Colletotrichum*, respectively, are known as pathogens and are also common components of foliar endophytes (Okane et al. 1998; Sahashi et al. 2000; Hata et al. 2002; Osono and Mori 2005; Osono 2008). Saikkonen et al. (2003) recorded *Fusicladium betulae* (an anamorph of *Ventulia ditricha*) and *Gnomonia setacea* as foliar endophytes of young trees of *Betula ermanii* and *B. platyphylla* planted in an experimental field in Finland. In the present study, *Gnomonia* sp. 11Af11 and *Ventulia* sp. 8Be61, closely related to *G. setaea* and *V. tremulae*

var. *tremulae*, respectively, were isolated from the same host trees in subalpine forest (Table 2). *Cladosporium* and *Khuskia* (including its anamorph *Nigrospora*) (Table 2) are known well as primary saprobes on tree leaves (Hudson 1968; Osono et al. 2009).

By compared endophytic fungal assemblages on leaves of phylogenetically diverse plant species collected from different climatic regions, Arnold and Lutzoni (2007) found that the endophyte diversity decreased linearly from the tropics to northern boreal forest. In the present study, such a climatic gradient was not found in the number of OTU of endophytic fungi on leaves of Betulaceae from subalpine, temperate, and subtropical forests, because of considerable variations between tree species within each forest type (Table 1). By contrast, the similarity of fungal OTU composition among the climatic regions was generally low (Table 4), which is consistent with Arnold and Lutzoni (2007) reporting that the similarities of fungal species composition were generally low between the climatic regions. It should be noted, however, that the differences in OTU composition between the climatic regions in the present study were attributable to not only climatic factors but also factors related to difference in plant species of the same genus (i.e., Table 5). Further studies are thus needed to evaluate the relative importance of climatic condition and host plant on endophytic fungal assemblages.

Previous studies of foliar endophytes on a few tree species reported two types of endophytic fungal assemblages in the cool temperate forest similar to the groups 1, 2, and 3 demonstrated in the present study. That is, Osono and Mori (2003) reported the frequent occurrence of Xylariaceous endophytes on *Fagus crenata* leaves, whereas Osono and Mori (2004, 2005) reported the frequent occurrence of *Colletotrichum* (an anamorph of *Glomerella*) on *Swida controversa* leaves. Osono (2012) also showed that 73 deciduous tree species in the study site were arranged along a continuum with these two types of foliar fungal assemblages at the ends. Reasons for the continuity of the endophytic fungus-tree leaf relationship remain unclear but would possibly be related to physical and chemical properties of leaves, microenvironmental conditions of phyllosphere, and patterns of leaf phenology.

Endophytic fungal assemblages on leaves of Betulaceae in subalpine forest showed relatively minor seasonal changes (Pianka's similarity index = 0.82), compared to those in cool temperate forest (0.00 to 0.19) (Table 6). Seasonal changes were also reported for endophytic fungi on leaves of *Swida controversa* studied in the same cool temperate forest (Osono and Mori 2005). The lower mean temperature and the lower variation in air temperature during the growing season of subalpine forest (Osono and Takeda 2007) may

partly account for the relatively minor changes in fungal OTU composition, compared to those in cool temperate forest. Seasonal dynamics of endophytic fungi in subtropical forest deserve future studies in this respect, as the mean temperature is higher but the seasonal variation in temperature was relatively lower than in temperate regions.

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Figure legend

**Fig. 1.** Principal components of endophytic fungal assemblages (a) and component loadings of major OTUs (b). Symbols are indicated with the combination of tree species and month; for example, Bg-N indicates *Betula grossa* in November. Tree species are as in Table 1. Mu, *Muscodor* sp. 11Bg52; Ne, *Nemania* sp. 8Cs51; Gn, *Gnomonia* sp. 11Af11; Ga, *Glomerella acutata*; Ap, *Apiosporopsis* sp. 11Af21; As, *Asteroma* sp. 8Ah91; Cc, *Cladosporium cladosporioides*; Kh, *Khuskia* sp. 11Bg11; Ni, *Nigrospora* sp. 11Bg31; My, *Mycosphaerella* sp. 8Ah31; Pe, *Pestalotiopsis* sp. 8Ch61.

# Fig . 1

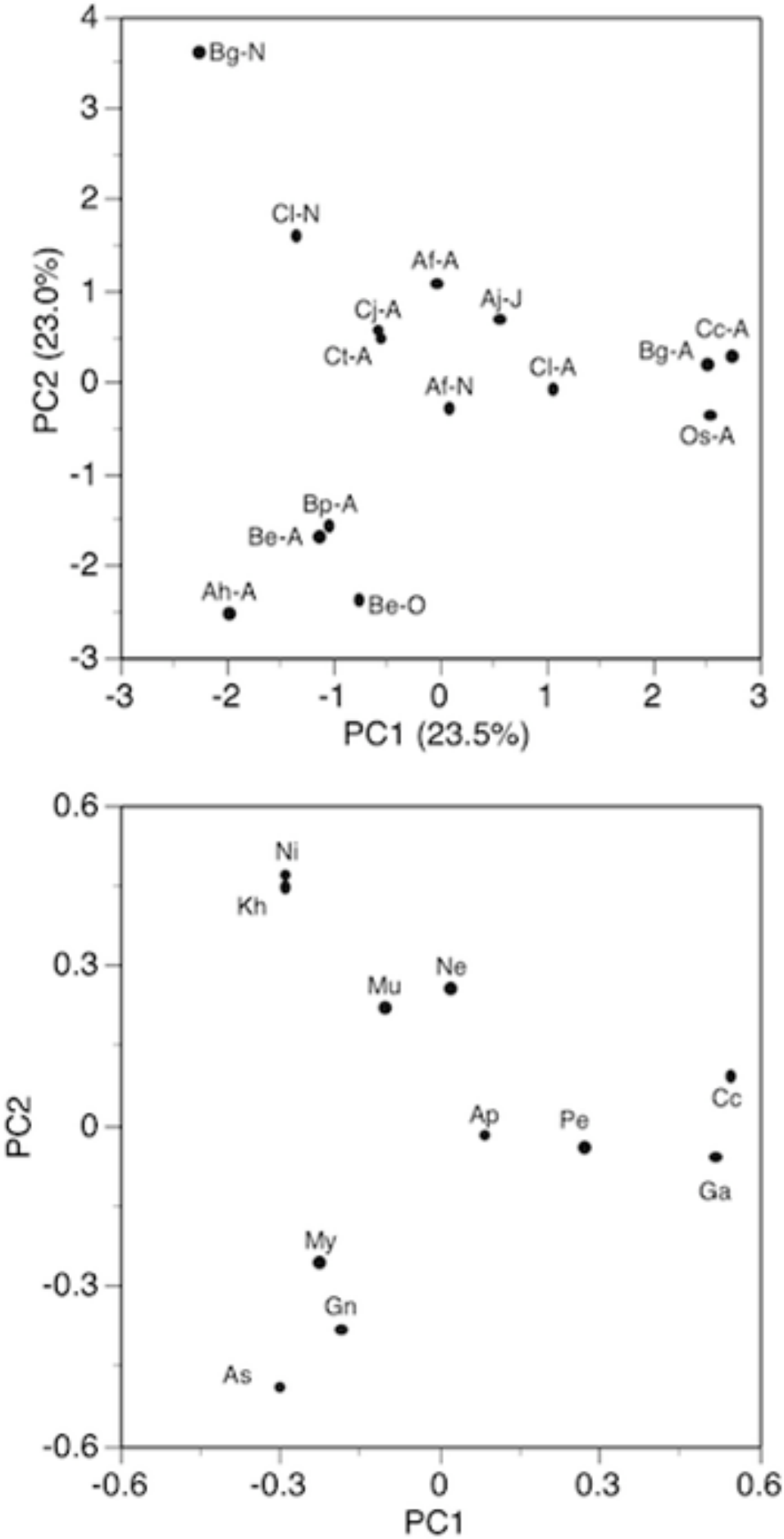

**Table 1.** Tree species, month of collection, and number of isolates and operational taxonomic units (OTU) of endophytic fungi.

| Tree species | Abbr. | Month in 2008 | Number of leaves examined | Number of isolates | Number of OTU |
|---|---|---|---|---|---|
| **Subalpine forest** | | | | | |
| *Alnus hirsuta* var. *sibirica* | Ah | Aug | 10 | 12 | 7 |
| *Betula ermanii* | Be | Jun | 10 | 0 | 0 |
| | | Aug | 10 | 10 | 5 |
| | | Oct | 10 | 12 | 4 |
| *Betula platyphylla* var. *japonica* | Bp | Aug | 10 | 11 | 5 |
| **Cool temperate forest** | | | | | |
| *Alnus fima* | Af | May | 10 | 2 | 1 |
| | | Aug | 10 | 14 | 9 |
| | | Nov | 10 | 11 | 3 |
| *Betula grossa* | Bg | May | 10 | 4 | 3 |
| | | Aug | 10 | 12 | 6 |
| | | Nov | 10 | 17 | 9 |
| *Corylus sieboldiana* | Os | Aug | 10 | 13 | 6 |
| *Carpinus laxiflora* | Cl | May | 10 | 2 | 2 |
| | | Aug | 10 | 6 | 5 |
| | | Nov | 10 | 16 | 13 |
| *C. tschonoskii* | Ct | Aug | 10 | 8 | 2 |
| *C. japonica* | Cj | Aug | 10 | 10 | 4 |
| *C. cordata* | Cc | Aug | 10 | 13 | 7 |
| **Subtropical forest** | | | | | |
| *Alnus japonica* | Aj | Jul | 10 | 13 | 7 |
| Total | | | 190 | 186 | |

2 **Table 2.** Blast search result of 46 operational taxonomic units (OTU) of endophytic fungi isolated from leaves of Betulaceae in Japan.

| Blast search result | Accession No. | Score (%) | Number of isolates | OTU | Accession No. of OTU |
|---|---|---|---|---|---|
| *Muscodor fengyangensis* | HM034862 | 99 | 21 | *Muscodor* sp. 11Bg52 | AB669030 |
| Fungal sp. mh337.6 | GU55255 | 98 | 20 | *Nemania* sp. 8Cs51 | AB669031 |
| *Gnomonia setacea* | AF277135 | 98 | 17 | *Gnomonia* sp. 11Af11 | AB669032 |
| *Glomerella acutata* | DQ286223 | 99 | 16 | *Glomerella acutata* | AB669033 |
| *Apiosporopsis carpinea* | AF277130 | 98 | 14 | *Apiosporopsis* sp. 11Af21 | AB669034 |
| *Asteroma alneum* | EU167609 | 98 | 8 | *Asteroma* sp. 8Ah91 | AB669035 |
| *Davidiella tassiana* | AY251078 | 99 | 8 | *Cladosporium cladosporioides* | AB669036 |
| *Khuskia* sp. | FJ890387 | 95 | 6 | *Khuskia* sp. 11Bg11 | AB669037 |
| *Nigrospora* sp. | EU852533 | 99 | 6 | *Nigrospora* sp. 11Bg31 | AB669038 |
| *Mycosphaerella berberidis* | EU167603 | 98 | 5 | *Mycosphaerella* sp. 8Ah31 | AB669039 |
| *Pestalotiopsis photiniae* | DQ657877 | 98 | 5 | *Pestalotiopsis* sp. 8Ch61 | AB669040 |
| *Botryosphaeria dothidea* | EU673243 | 98 | 4 | *Botryosphaeria* sp. 5Af11 | AB669041 |
| *Phomopsis* sp. | EU219393 | 98 | 4 | *Phomopsis* sp. 8Af32 | AB669042 |
| *Biscogniauxia* sp. | DQ840054 | 97 | 3 | *Biscogniauxia* sp. 8Bg12 | AB669043 |
| Xylariaceae sp. | DQ674832 | 98 | 3 | *Annulohypoxylon* sp. 8Af31 | AB669044 |
| *Annulohypoxylon moriforme* | DQ840058 | 99 | 3 | *Annulohypoxylon* sp. 8Bp31 | AB669045 |
| *Glomerella cingulata* | DQ286193 | 99 | 3 | *Glomerella cingulata* | AB669046 |
| *Daldinia childiae* | EF562505 | 95 | 3 | *Daldinia* sp. 11Bg22 | AB669047 |
| *Rosellinia corticium* | DQ840078 | 98 | 3 | *Rosellinia* sp. 8Af62 | AB669048 |
| *Venturia tremulae* var. *tremulae* | EU035475 | 99 | 3 | *Venturia* sp. 8Be61 | AB669049 |
| Fungal endophyte isolate 9202 | EF420079 | 95 | 2 | *Xylaria* sp. 11Bg53 | AB669050 |
| *Gnomonia rostellata* | EF212860 | 97 | 2 | *Gnomonia* sp. 7Aj71 | AB669051 |
| *Pestalotiopsis* sp. AJH26 | EU605882 | 94 | 2 | *Pestalotiopsis* sp. 11Cl72 | AB669052 |
| *Thuemenella cubispora* | EF562509 | 95 | 2 | *Thuemenella* sp. 8Af22 | AB669053 |

| | | | | | |
|---|---|---|---|---|---|
| *Chlorencoelia* sp. | AY789351 | 93 | 2 | *Chlorencoelia* sp. 8Bg11 | AB669054 |
| *Arthrinium phaeospermum* | AY083832 | 98 | 1 | *Arthrinium phaeospermum* | AB669055 |
| *Botryosphaeria parva* | AY928046 | 99 | 1 | *Botryosphaeria* sp. 8Af12 | AB669056 |
| *Chaetomium bostrychodes* | AF286395 | 96 | 1 | *Chaetomium* sp. 11Cl21 | AB669057 |
| *Coniochaeta velutina* | EU999180 | 96 | 1 | *Coniochaeta* sp. 11Af32 | AB669058 |
| *Diaporthe eres* | AF362565 | 99 | 1 | *Diaporthe* sp. 8Cs61 | AB669059 |
| *Diaporthe acaciigena* | DQ377874 | 91 | 1 | *Diaporthe* sp. 11Cl22 | AB669060 |
| *Didymella fabae* | FJ755246 | 93 | 1 | *Didymella fabae* | AB669061 |
| Uncultured Pleosporales | EU490012 | 91 | 1 | Dothideomycetes sp. 11Bg71 | AB669062 |
| *Allantophoma endogenospora* | EU754126 | 94 | 1 | *Gnomonia* sp. 11Bg1 | AB669063 |
| *Penicillium verruculosum* | AF510496 | 99 | 1 | *Penicillium verruculosum* | AB669064 |
| *Pestalotiopsis* aff. *palmarum* | FJ890415 | 97 | 1 | *Pestalotiopsis* sp. 11Bg91 | AB669065 |
| Uncultured Leotiomycetes | JF449482 | 97 | 1 | *Phialophora* sp. 5Bg41 | AB669066 |
| *Phomopsis* sp. | AB107264 | 90 | 1 | *Phomopsis* sp. 11CL13 | AB669067 |
| Uncultured soil fungus | EU692696 | 96 | 1 | fungal sp. 8Ah82 | AB669068 |
| *Pseudoplectania nigrella* | AY945852 | 91 | 1 | fungal sp. 5Cl21 | AB669069 |
| *Anthostomella leucospermi* | EU552100 | 95 | 1 | *Xylaria* sp. 8Cs12 | AB669070 |
| *Biscogniauxia nummularia* | GQ428318 | 96 | 1 | fungal sp. 8Ci42 | AB669071 |
| *Cryptodiaporthe aesculi* | DQ836905 | 98 | 1 | *Cryptodiaporthe* sp. 8Ah92 | AB669072 |
| *Harknessia gibbosa* | EF110615 | 93 | 1 | *Harknessia* sp. 11Af41 | AB669073 |
| *Trimmatostroma salicis* | EU019300 | 96 | 1 | *Trimmatostroma* sp. 8Cl62 | AB669074 |
| *Coniothyrium nitidae* | EU552112 | 96 | 1 | *Coniothyrium* sp. 8Cl93 | AB669075 |

5

**Table 3.** Frequency of occurrence (%) of major five OTUs of endophytic fungi on leaves of Betulaceae in Japan. Tree species are as in Table 1.

| Tree species | Month in 2008 | *Muscodor* sp. 11Bg52 | *Nemania* sp. 8Cs51 | *Gnomonia* sp. 11Af11 | *Glomerella acutata* | *Apiosporopsis* sp. 11Af21 |
|---|---|---|---|---|---|---|
| **Subalpine forest** | | | | | | |
| Ah | Aug | 0 | 10 | 0 | 0 | 0 |
| Be | Jun | 0 | 0 | 0 | 0 | 0 |
| | Aug | 0 | 10 | 40 | 0 | 0 |
| | Oct | 0 | 0 | 70 | 20 | 0 |
| Bp | Aug | 10 | 0 | 60 | 0 | 0 |
| **Cool temperate forest** | | | | | | |
| Af | May | 0 | 0 | 0 | 0 | 0 |
| | Aug | 50 | 10 | 0 | 0 | 0 |
| | Nov | 0 | 0 | 0 | 0 | 90 |
| Bg | May | 0 | 10 | 0 | 0 | 0 |
| | Aug | 0 | 20 | 0 | 40 | 10 |
| | Nov | 20 | 30 | 0 | 0 | 0 |
| Os | Aug | 0 | 0 | 0 | 30 | 20 |
| Cl | May | 0 | 0 | 0 | 0 | 10 |
| | Aug | 10 | 0 | 0 | 20 | 0 |
| | Nov | 0 | 0 | 0 | 0 | 10 |
| Ct | Aug | 70 | 0 | 0 | 0 | 0 |
| Cj | Aug | 40 | 40 | 0 | 0 | 0 |
| Cc | Aug | 10 | 20 | 0 | 50 | 0 |
| **Subtropical forest** | | | | | | |
| Aj | Jul | 0 | 50 | 0 | 0 | 0 |

**Table 4.** Pianka's similarity index for endophytic fungal assemblages on leaves among subalpine, cool temperate, and subtropical tree species. Tree species are as in Table 1. na data not available.

| Tree genus | Tree species | Month | Subalpine vs cool temperate | Subalpine vs subtropic | Cool temperate vs subtropic |
|---|---|---|---|---|---|
| *Alnus* | Ah, Af, Aj | Jul-Aug | 0.03 | 0.15 | 0.30 |
| *Betula* | Be, Bg | Aug | 0.07 | na | na |
| | | Oct-Nov | 0.02 | na | na |
| | Bp, Bg | Aug | 0.00 | na | na |

**Table 5.** Pianka's similarity index for endophytic fungal assemblages on leaves among tree species. Tree species are as in Table 1.

**(1) Subalpine forest, August**

| | Ah | Be | Bp |
|---|---|---|---|
| Ah | | 0.32 | 0.08 |
| Be | | | 0.84 |
| Bp | | | |

**(2) Cool temperate forest, August**

| | Af | Ct | Cj | Bg | Cl | Cc | Os |
|---|---|---|---|---|---|---|---|
| Af | | 0.82 | 0.69 | 0.12 | 0.35 | 0.25 | 0.05 |
| Ct | | | 0.68 | 0.00 | 0.35 | 0.16 | 0.00 |
| Cj | | | | 0.25 | 0.24 | 0.34 | 0.00 |
| Bg | | | | | 0.65 | 0.84 | 0.46 |
| Cl | | | | | | 0.76 | 0.39 |
| Cc | | | | | | | 0.46 |
| Os | | | | | | | |

**(3) Cool temperate forest, November**

| | Af | Bg | Cl |
|---|---|---|---|
| Af | | 0.00 | 0.21 |
| Bg | | | 0.52 |
| Cl | | | |

**Table 6.** Pianka's similarity index for endophytic fungal assemblages on leaves among seasons. Tree species are as in Table 1. na data not available.

| Forest type | Tree species | May-Jun vs Aug | May-Jun vs Oct | Aug vs Oct-Nov |
|---|---|---|---|---|
| Subalpine | Be | na | na | 0.82 |
| Cool temperate | Af | 0.00 | 0.00 | 0.00 |
| | Bg | 0.15 | 0.19 | 0.17 |
| | Cl | 0.00 | 0.15 | 0.00 |